Normal tension glaucoma (NTG) is a subtype of glaucoma that presents with statistically normal intraocular pressure (IOP), suggesting the possibility that non-IOP-dependent factors may contribute to the disease progression. We previously reported that loss of glutamate transporters (excitatory amino acid carrier 1 [EAAC1] or glutamate/aspartate transporter [GLAST]) in mice leads to progressive retinal ganglion cell (RGC) loss and optic nerve degeneration while maintaining normal IOP, demonstrating pathological features that are similar to NTG. EAAC1 is expressed on RGCs and transports glutamate into cells together with cysteine and glycine, which are converted to glutathione, a major antioxidant and free radical scavenger, and immunohistochemical analyses of phosphorylated p38 mitogen-activated protein kinase (MAPK) in the retina were performed at 8 weeks old.

RESULTS. Topical ripasudil ameliorated retinal degeneration and improved visual function in EAAC1 KO mice at both 8 and 12 weeks old. Ripasudil reduced IOP and strongly suppressed the phosphorylation of p38 MAPK that stimulates RGC death in EAAC1 KO mice.

CONCLUSIONS. These results suggest that, in addition to IOP reduction, ripasudil prevents glaucomatous retinal degeneration by neuroprotection, which is achieved by suppressing cell-death signaling pathways.

Keywords: ripasudil, glaucoma, neuroprotection, intraocular pressure, p38
present study, we found that topical ripasudil prevents glaucomatous retinal degeneration in EAAC1 KO mice by stimulating an IOP-independent pathway, in addition to IOP-dependent pathways.

**Materials and Methods**

**Mice**

Experiments were performed using EAAC1 KO mice (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) on a C57BL6 background, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Drug Administration**

From 5 weeks of age (5 W) to 8 or 12 W, EAAC1 KO mice received once-daily topical administration (5 µL/day) of phosphate-buffered saline (PBS) as the control or two different concentrations of ripasudil (Kowa Pharmaceutical Co. Ltd., Tokyo, Japan) solution: 0.4% and 2% in PBS. We selected these doses of ripasudil because 0.4% is the clinical concentration and 2% is the concentration that can be stably dissolved without precipitation at normal temperature.

**IOP Measurement**

IOP was measured by a commercial rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH, USA) in anesthetized mice, as reported previously. To minimize variation, data were collected between 15:00 and 18:00, 4 to 6 minutes after injection of the anesthetic, during which time IOP plateaus. IOP was measured at 5, 8, and 12 W.

**Imaging Acquisition of Spectral-Domain Optical Coherence Tomography (SD-OCT)**

SD-OCT (RS-3000; Nidek, Aichi, Japan) examinations were performed at 5, 8, and 12 W. For fundus imaging, polymethyl methacrylate contact lenses optimal for mice (UNICON, Osaka, Japan) were placed on the corneas. The lenses prevent anesthesia-induced cataract progression. A 60-D adaptor lens was placed on the objective lens of the Multiline OCT to focus on the retina. All line scan images were location matched, scanning vertically through the center of the optic nerve head at three-disc diameter lengths above it. The average thickness of the ganglion cell complex (GCC; between the internal limiting membrane and the interface of the inner plexiform layer and the inner nuclear layer) and the layers other than GCC were measured. The maximum number of B-scans set by the manufacturer (50) was used for averaging.

**Histological and Morphometric Studies**

Mice were perfused with Zamboni’s fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer) at 5, 8, and 12 W. Eyes were enucleated and postfixed in 3% glutaraldehyde solution (3% glutaraldehyde, 9% formaldehyde, 37.5% ethanol, and 12.5% acetic acid in distilled water) for 2 hours. Paraffin-embedded retinal sections of 7-µm thickness were cut through the optic nerve and stained with hematoxylin and eosin (H&E). The RGC number and the extent of retinal degeneration were quantified in two ways. First, the number of neurons in the ganglion cell layer (GCL) of the retina was counted from one ora serrata through the optic nerve to the other ora serrata. Second, in the same section, the thickness of the IRL (between the internal limiting membrane and the interface of the outer plexiform layer with the outer nuclear layer) was measured.

**Retrograde Labeling**

Mice were deeply anesthetized with isoﬂurane (Intervet, Tokyo, Japan), placed on a stereotaxic frame, and injected with 2 µL of 1% Fluoro-Gold (FG; Fluorochrome LLC, Denver, CO, USA) dissolved in PBS into the superior colliculus. Ten days after FG application, mice were anesthetized, the eyes were enucleated, and the retinas were isolated for whole mount preparation. The retinas were fixed in 4% paraformaldehyde in PBS for 20 minutes and mounted on a glass slide with a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), and the RGC density was examined with a fluorescent microscope. The excitation and emission wavelengths for FG were 323 and 620 nm, respectively. One central (0.1 mm from the optic disc) and one peripheral (1.0 mm from the optic disc) areas (0.04 mm²) per quadrant of each retina were chosen. FG-labeled cells were manually counted, and the mean number of RGCs per square millimeter was calculated.

**Multifocal Electroretinogram (mfERG)**

Mice were anesthetized at 5, 8, and 12 W by intraperitoneal injection of 87.5 mg/kg sodium pentobarbital. The pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. The mfERGs were recorded using a VERIS 6.0 system (Electro-Diagnostic Imaging, Redwood City, CA, USA). The visual stimulus consisted of seven hexagonal areas scaled with eccentricity. The stimulus array was displayed on a high-resolution black-and-white monitor with a frame rate of 100 Hz. The second-order kernel, which is impaired in patients with glaucoma, was analyzed as previously reported.

**Immunoblot Analyses**

Immunoblotting was performed as previously reported. Membranes were incubated with an antibody against p38 (1:1000; sc-535; Santa Cruz, Santa Cruz, CA, USA) or phosphorylated p38 (1:1000; 612280; BD Biosciences, San Jose, CA, USA). The intensities were analyzed using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry**

Mice were perfused with Zamboni’s fixative at 12 W. Eyes were enucleated, postfixed in Zamboni’s fixative for 2 hours, and then transferred into a sucrase buffer (30% sucrose in a 0.1-M phosphate buffer) for cryoprotection. Retinal cryostat sections of 10-µm thickness were prepared and examined by immunostaining using an antibody against RBPG (1:1000; ab194213; Abcam, Cambridge, MA, USA), glutamine synthetase (GS; 1:1000; MAB302; Merck Millipore, Billerica, MA, USA), calretinin (1:1000; 66496-1-Ig; Proteintech, Chicago, IL, USA), protein kinase C (PKC; 1:1000; 21991-1-AP; Proteintech), calbindin (1:1000; 14479-1-AP; Proteintech), or phosphorylated p38 (1:1000). The intensities of phosphorylated p38 at the GCL were analyzed using ImageJ.

**Statistics**

Data are presented as mean ± SEM. When statistical analyses were performed, the Student’s t-test or 1-way ANOVA followed by a Tukey’s test was used. P < 0.05 was regarded as
statistically significant. JMP version 13.1.0 (SAS Institute, Inc., Cary, NC, USA) was used for the statistical analyses.

RESULTS

NTG-like Retinal Degeneration in EAAC1 KO Mice

We previously reported progressive RGC loss from 5 to 12 W in EAAC1 KO mice. Consistently, the RBPMS-positive RGC number was significantly decreased in EAAC1 KO mice compared with wild-type (WT) mice at 12 W (Figs. 1A, 1B). To examine the effects of EAAC1 on other retinal cell types, we carried out immunohistochemistry with calretinin (a marker of RGCs and amacrine cells), calbindin (a marker of horizontal cells), PKC (a marker of bipolar cells), or GS (a marker of Müller glial cells), but we could detect no differences in their expression patterns between WT and EAAC1 KO mice (Fig. 1A).

We also visualized the retinal layers in living mice using SD-OCT, a noninvasive imaging technique that can be used to acquire cross-sectional tomographic images of the retina. For quantitative analysis, GCC was measured by scanning the retina in a circle centering around the optic nerve disc (Fig. 1C), and the average GCC thickness was determined from acquired images (Fig. 1D). The average thickness of the GCC, which contains the nerve fiber layer, GCL, and inner plexiform layer, was significantly smaller in EAAC1 KO mice compared with WT mice (Fig. 1E). Whereas, the thickness of the other retinal layers was similar between WT and EAAC1 KO mice (Fig. 1F). These data reconfirmed our previous findings showing NTG-like RGC degeneration in EAAC1 KO mice.

Effects of Ripasudil on IOP in EAAC1 KO Mice

In this study, we topically administered daily ripasudil (0.4% or 2%) or PBS as a control to EAAC1 KO mice from 5 W through to 8 or 12 W (Fig. 2A) to investigate whether it is capable of preventing NTG-like phenotypes. First, we examined the effects of ripasudil on IOP. We previously reported that the...
FIGURE 2. Experimental protocols and effects of ripasudil on IOP. (A) Experimental protocols. Ripasudil (0.4% or 2%; 5 μL) or PBS (5 μL) was administered locally every day from 5 W. The mice were euthanized at 5, 8, or 12 W. (B) IOP at 5, 8, and 12 W. The data are presented as mean ± SEM; there are 12 samples for 5 and 8 W and six samples for 12 W. *P < 0.05, **P < 0.01.

FIGURE 3. In vivo imaging of the retina in EAAC1 KO mice treated with ripasudil. (A) OCT cross-sectional images of retinas at 5, 8, and 12 W. (B) Longitudinal evaluation of the GCC thickness by a circular scan. The data are presented as mean ± SEM; there are 12 samples for 5 and 8 W and six samples for 12 W. **P < 0.01, ***P < 0.001.
IOP of EAAC1 KO mice was not significantly increased compared with WT mice at 5, 8, and 12 W. At both concentrations, ripasudil slightly but significantly reduced IOP in EAAC1 KO mice compared with the control (Fig. 2B). There was no significant difference between the two concentrations.

Ripasudil Protects RGCs in EAAC1 KO Mice

We next examined the GCC thickness at 8 and 12 W in EAAC1 KO mice treated with 0.4% and 2% ripasudil using SD-OCT. GCC thickness was greater in ripasudil-treated EAAC1 KO mice than control EAAC1 KO mice (Fig. 3A). Quantitative analysis showed that GCC thickness at 8 and 12 W was significantly decreased in control mice, but it was almost unaltered in mice treated with 0.4% and 2% ripasudil (Fig. 3B). There was no significant difference between the two concentrations. These data indicate that both concentrations protect RGCs from NTG-like neurodegeneration.

We previously reported that the cell number in the GCL of EAAC1 KO mice at 8 and 12 W was significantly lower than WT mice. In addition, the thickness of the IRL in EAAC1 KO mice was significantly reduced at 8 and 12 W. The number of surviving neurons in EAAC1 KO mice treated with 0.4% or 2% ripasudil was significantly greater than that in control mice at 8 and 12 W (Figs. 4A, 4B). In addition, both concentrations of ripasudil treatment prevented the thinning of the IRL (Fig. 4C). There was no significant difference between the two concentrations. Because GCL contains cell types other than RGCs, including displaced amacrine cells, we next performed retrograde labeling of RGCs with FG on control and ripasudil-treated mice at 8 W. In this experiment, we used the lower dose only because the neuroprotective effect was similar between 0.4% and 2% (Fig. 4). Consistent with the results of cell counting in the GCL (Fig. 4B), the RGC number in ripasudil-treated EAAC1 KO mice was significantly higher than control mice in both the central and peripheral regions (Fig. 5). These data demonstrate that topical ripasudil prevents RGC death all across the retina in EAAC1 KO mice.

Ripasudil Ameliorates Visual Impairment in EAAC1 KO Mice

To determine if the histological observation of ripasudil-mediated neuroprotection in EAAC1 KO mice reflects functional aspects, we examined retinal function using mfERG. We analyzed the second-order kernel component, which appears to be a sensitive indicator of inner retinal dysfunction and is
mice are functionally significant.

These results confirm that the neuroprotective effects of ripasudil on glaucomatous retinal degeneration in EAAC1 KO mice, but ripasudil treatment significantly suppressed the ratio (Fig. 7B). We observed no marked changes in the protein levels of the total p38 in any groups. We also performed immunohistochemical analysis of phosphorylated p38 in the mouse retina at 8 W. Phosphorylated p38 was mainly observed in the GCL of EAAC1 KO mice, and it was barely detected in WT mice or ripasudil-treated EAAC1 KO mice (Fig. 7C). Quantitative analyses confirmed that the phosphorylation of p38 is significantly suppressed with ripasudil treatment in EAAC1 KO mice (Fig. 7D).

**DISCUSSION**

In this study, we showed that topical administration of ripasudil reduced the IOP of EAAC1 KO mice. We also showed that phosphorylation of p38, which is indicative of increased oxidative stress, is reduced in EAAC1 KO mice with ripasudil treatment. Consistently, ripasudil prevented progressive RGC death, thinning of the IRL, and impairment in retinal function in EAAC1 KO mice. To monitor these changes in the same animal, we used OCT and mfERG that permit in vivo, noninvasive, quantitative assessments of the changes in retinal morphology and function in EAAC1 KO mice. These techniques clearly visualized the therapeutic effects of ripasudil and provide useful information in experimental animals and for clinical trials and management. We tested two concentrations, 0.4% and 2%, but there were no differences in the therapeutic effects, suggesting that the lower concentration was sufficient to evoke the maximum therapeutic response. We demonstrated that the topical administration of ripasudil reduces IOP and the activation of p38 in EAAC1 KO mice, which leads to suppression of glaucomatous neurodegeneration.

ROCK inhibitors may be drug candidates for neuroprotection as well as lowering IOP. For example, fasudil attenuated the ischemia/reperfusion-induced apoptosis of retinal cells independent of changes in IOP. We previously reported that brimonidine exerted neuroprotective effects on glaucomatous degeneration in EAAC1 KO mice. Brimonidine is known to lower IOP and have neuroprotective effects in glaucoma patients. Since ripasudil showed a similar reduction rate of IOP and neuroprotection in EAAC1 KO mice, we think it is plausible that ripasudil also prevents RGC death through both IOP-dependent and IOP-independent pathways.

We previously reported that traumatic optic nerve injury activates p38 and that intracocular injection of a p38 inhibitor prevents RGC death. In this study, we detected phosphorylated p38 in the GCL of EAAC1 KO mice independent of IOP, but topical ripasudil significantly suppressed its activity (Fig. 7). These findings are consistent with the previous reports that other ROCK inhibitors, such as fasudil and H1152, suppressed stress-activated MAPK family members (p38 and c-Jun N-terminal kinase [JNK]). The inhibition of p38 has been shown to be potentially beneficial in experimental nerve trauma, excitotoxicity, and growth factor withdrawal. As a result, p38 inhibitors have recently been claimed as novel and potential therapeutics for neurodegenerative diseases. These findings suggest that inhibition of p38 may be therapeutically effective in protecting degenerating RGCs from various pathological conditions, including glaucoma.

ROCK inhibitors also have vascular smooth muscle relaxant action and were originally used to treat vasospasm cerebral infarction after subarachnoid hemorrhage. ROCK inhibitors increase the blood flow in the optic nerve head. Decreased blood flow to the eye is one of the risk factors of glaucoma. In addition, ripasudil may suppress TNF-induced optic nerve...
degeneration by modulating autophagy, so ripasudil may suppress glaucoma through multiple neuroprotective effects.

In this study, we have used EAAC1 KO mice as an animal model of NTG. This model closely mimics pathology of NTG, including RGC loss, optic nerve atrophy, and visual impairment while maintaining normal IOP. However, there are some limitations to using mouse models. For example, the RGC loss in EAAC1 KO mice is distributed across the entire retina, rather than in specific regions as seen in human glaucoma and in some mouse models of ocular hypertension induced glaucoma, and retinal degeneration in EAAC1 KO mice starts at 5 weeks of age, earlier and faster time-course than one may expect from human glaucoma. Despite some limitations, such animal models are essential for preclinical research and our NTG models have been providing useful information regarding NTG therapy easily and speedily. We recently reported that widely prescribed drugs, such as valproic acid, candesartan, and edaravone, suppressed RGC death in EAAC1 or GLAST KO mice without altering IOP. In addition, every other day fasting, a form of caloric restriction, suppressed RGC death in EAAC1 KO mice under normal IOP. These findings raise intriguing possibilities for the management of glaucoma by caloric restriction and/or utilizing existing drugs for neuroprotection in combination with conventional treatments to lower IOP. Further studies will be required to determine the long-term effect of ripasudil on p38 activation and ocular blood flow in EAAC1 KO mice and other animal models of glaucoma.

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**FIGURE 7.** Effects of 0.4% ripasudil on phosphorylation of p38 in the retinas in EAAC1 KO mice. (A) Immunoblot analysis of phosphorylated p38 and total p38 in the retinas of WT and EAAC1 KO mice at 5 W. Ratio of p38 phosphorylation in WT mice was estimated as 100%. (B) Immunoblot analysis of phosphorylated p38 and total p38 in the retinas of WT, PBS- and ripasudil-treated EAAC1 KO mice at 8 W. Ratio of p38 phosphorylation in WT mice was estimated as 100%. (C) Representative images of phosphorylated p38 in the retina at 8 W. (D) Quantitative analyses of (C). The phosphorylated p38 intensity at the GCL in WT mice was estimated as 100%. The data are presented as mean ± SEM of six samples for each experiment. *P < 0.05, ** P < 0.01.


